PERIPHERAL BLOOD CELL DNA METHYLATION AND HYDROXYMETHYLATION IN PROSTATE CANCER. A PILOT STUDY

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ABSTRACT

Objective/Purpose: DNA methylation is an important epigenetic hallmark of cancer, including prostate cancer, with a general pattern of global hypomethylation accompanied by hypermethylation at certain gene promoters' level in tissues. Limited evidence exists on the methylation pattern of blood cells in prostate cancer patients, which this pilot study is addressing. **Material and Methods:** DNA methylation and hydroxymethylation levels were assessed in peripheral nucleated blood cells from prostate cancer patients compared to controls using fluorimetric ELISA-type assays that directly measure the amount of 5mC and 5hmC, respectively. **Results:** There was significant difference in global DNA methylation levels between prostate cancer patients and controls, with almost two-fold hypomethylation observed in patients' blood cell DNA (p<0.0001). There was no statistical significant difference regarding the hydroxymethylation levels. **Conclusions:** In this pilot study of limited sample sizewe found evidence of different levels of global DNA methylation in blood cells of prostate cancer patients compared to controls. These findings need further replication in future larger studies in order to assess the biomarker potential of blood cells DNA methylation and hydroxymetylation in prostate cancer.
Keywords: DNA methylation, DNA hydroxymethylation, prostate cancer, peripheral blood cells

INTRODUCTION

According to the American Cancer Society's most recent report, prostate cancer is the second leading cause of cancer related death in men[1].For many years, cancer research efforts are focused towards the development of non-invasive tests for screening, diagnosis and management of cancer, which have the potential of reducing the health burden of this disease worldwide [2]. Current circulating biomarkers are mainly protein-based and lack in sensitivity and/or specificity [3]. To date, PSA is the most frequently used biomarker for prostate cancer in clinical settings; however, its usefulness has come under severe scrutiny recently because of its increased sensitivity, low specificity and minimal effect on prostate cancer mortality[4]. Consequently, further research is needed in order to develop new non-invasive markers for prostate cancer, that could complement the ones currently in use.

Recent advances in understanding the molecular mechanisms governing prostate cancer initiation and progression include the epigenetic changes that influence gene expression that in turn promotes malignant transformation by assisting in tumor invasion, angiogenesis, and cellular dedifferentiation and proliferation[5][6]. These epigenetic changes include DNA methylation, histone post-translational modifications, and non-coding RNAs. Among these, particular interest has been devoted in recent years to micro RNAs due to their stability in biological fluids, that makes them good candidates for biomarkers in biological fluids such as serum/plasma and urine[7][8].

The study of DNA methylation in tissues has reached a mature stage, providing solid evidence of the role of this epigenetic modification in cancer development, including of prostate cancer. As in other cancers, the DNA methylation patterns of prostate cancer tissue compared to normal tissue include a hypomethylation at aglobal level accompanied by hypermethylation at certain gene promoters' level[9]. In addition to methylation on the cytosine's 5-carbon to form 5-methylcytosine (5mC), other cytosine modifications can occur leading to the formation of 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Although less known, these latter forms of modified cytosineappear to represent independent epigenetic marks, including in prostate cancer tissues[10]. Recent data suggests that hydroxymethylation is involved in regulating distinct biological pathways from methylation and can serve as a prognostic marker of recurrence in certain types of prostate cancer[11][12].

In contrast to DNA methylation studies in prostate cancer tissue, few studies have been focused on investigating the DNA methylation levels of peripheral DNA in prostate cancer patients as a possible biomarker. We describe herein a pilot study on the level of global DNA methylation and hydroxymethylation in peripheral nucleated blood cells (PNBCs) of prostate cancer patients compared to healthy controls.

MATERIAL AND METHODS

Study subjects

Patients diagnosed and treated for prostate cancer and subjects with benign diseases from the Urology Clinic of the Clinical Emergency County Hospital in Timisoara, Romania, were included in this study. All subjects provided informed consentand the study was approved by the Ethics Committees of the participating institutions. Prostate specific antigen (PSA) testing was performed in the Clinical Laboratory of the Timisoara Clinical Emergency County Hospital for cancer patients and healthy controls.

Biological samples

EDTA-treated blood collection tubes were used forvenous blood collection, which was immediately centrifuged for 15 minutes at 2000g for plasma and buffy coat separation which were then frozen at -80 degrees Celsius. Genomic DNA was extracted from the buffy coat PNBCs using the QIAmp DNA Mini kit (Qiagen), according to the manufacturer's instructions. After isolation, the DNA concentration and quality were assessed using a NanoDropMicrovolume Spectrophotometer (Thermo Fischer Scientific), and the samples were stored at -80 degrees Celsius.

DNA methylation and hydroxymethylation analysis

The quantification of global DNA methylation was assessed using the MethylFlash Methylated DNA Quantification Kit (Fluorometric) and hydroxymethylation was done using theMethylFlashHydroxymethylated DNA Quantification Kit (Fluorometric), both from Epigentek, according with the producer's protocols. These ELISA type assays quantify the amount of 5mC and 5hmC from the total amount of genomic DNA, respectively. The methylated and hydroxymethylated fractions of DNA were quantified fluorometrically by reading the RFU (relative fluorescence units) with a fluorescence spectrophotometer (GloMax Discover Multimode Microplate Reader, Promega) and data are presented as percent methylation and hydroxymethylation, respectively.

Statistical analysis

The D'Agostino-Pearson test was used to verify the normal distribution of the data. As a consequence, levels of methylation and hydroxymethylation in cancer and control subjects were compared using unpaired two tailed t statistics, and $p < 0.05$ was considered for statistical significance. Statistical analyses were done using the GraphPad Prism v.6.01. software.

RESULTS

Study subjects' characteristics

The clinical and demographic characteristics of the study participants are presented in **Table 1.** The prostate cancer patients included in the study were older compared to controls, had PSA levels higher than 4ng/ml and their prostate tumors had a Gleason score above 7 in most cases (85.71%).

Characteristics	Cases (N=14)	Controls (N=15)
Age (years±SD)	65.5 (±5.05)	51.0 (±8.09)
PSA N(%)		
<4 ng/ml	0 (0.00)	15 (100.00)
4-10 ng/ml	5 (35.71)	0 (0.00)
≥10 ng/ml	9 (64.28)	0 (0.00)
Gleason ScoreN(%)		
5-6	2 (14.28)	
7	10 (71.43)	
8-10	2 (14.28)	

Table 1.
Clinical and demographic characteristics of the study participants

DNA methylation and hydoxymethylation levels

The PNBCs DNA methylation and hydroxymethylation data is presented in **Table 2.**

	Cases	Controls	p val
DNA meth % (Mean±SD)	1.150±0.515	2.290±0.739	< 0.0001
DNA methOH % (Mean±SD)	0.022±0.016	0.028±0.015	0.3686

Table 2.
Methylation and hydroxymethylation data

The average global DNA methylation level was 1.15% in cases and 2.29% in controls. The almost two-fold methylation difference was statistically significant (p<0.0001), prostate cancer patients presenting marked hypomethylation in the PNBCs DNA compared to controls, as shown in **Figure 1.** The global DNA methylation in PNBCs was lower than 4% in all cases and controls, as seen in **Figure 1.**

The average global DNA hydroxymethylation level was similar in prostate cancer patients compared to controls (0.022% and 0.028%, respectively), with no statistical significant difference among them (Table 2 and Figure 2). The global DNA hydroxymethylation in PNBCs was lower than 0.07% in all cases and controls, as seen in Figure 2.

The ROC curve for DNA methylation is presented in Figure 3. The area under the curve (AUC) value was 0.904 (95%CI:0.799-1.01), which was lower than that for PSA (100% in our pilot study, data not

shown). At a selected cutoff value of 1.64% DNA methylation, the sensitivity was 78.57% and specificity was 80%.

Figure 1.
DNA methylation percent

Figure 2.
DNA hydroxymethylation percent

Figure 3.
ROC curve for DNA methylation

DISCUSSION

To our knowledge, this is the first study assessing together the levels of global DNA methylation and hydroxymethylation in PNBCs of prostate cancer patients compared to controls. Although this is a pilot study of limited samples size, we have confirmed the data presented in the only other study available showing that DNA hydorxymethylation of blood cells is no different between prostate cancer patients and controls[13]. This study used the same method as ours to determine the hydroxymethylation levels in blood cells DNA, and the values for prostate cancers and controls were similar; however, subjects with benign prostatic hyperplasia (BPH) and atypical small acinar proliferation (ASAP) had significantly higher levels of hydroxymethylation compared to controls. Unfortunately, we did not include subjects with BPH and ASAP in our study. Compared to normal prostate tissue, cancer tissue displays lower levels of 5hmC, which is regulated at least in part by ten-eleven translocation (TET) proteinsthat oxidize 5-methylcytosine to 5-hydroxymethylcytosine and further derivatives[14]. It appears that this pattern is localized to the prostate tissue and not mirrored by the PNBCs in prostate cancer patients.

The DNA hypermethylation at gene promoters level that could be promising novel tissue biomarkers is well documented in prostate cancer tissue compared to adjacent normal [15][16]. Limited evidence exists that hypermethylation at certain specific DNA regions is found in nucleated blood cells of prostate cancer patients. A recent study of 50 methylated sites at chromosome location 8q24 in DNA extracted from whole blood revealed that several of these were associated with prostate cancer, suggesting that the methylation of specific loci in blood DNA could serve as markers for prostate cancer [17].

Moreover, in addition to gene promoter hypermethylation, a global hypomethylation was observed in prostate cancer tissues compared to normal tissue, in line with data on other cancer types [18].Most studies assessing global DNA methylation levels use surrogate markers such as LINE-1 and Alu repeats which are distributed along the human genome and whose methylation levels represent the global methylation across the entire genome. Using this type of analysis, Han et al. found no association between LINE-1 methylation levels in blood leukocytes and high-risk prostate cancer or risk of recurrence in a case-only study, concluding that LINE-1 methylation in blood cells cannot be used as a biomarker of prostate cancer aggressiveness or prognosis [19]. However, another study found an association between Alu methylation and prostate cancer that varied by time from blood draw to diagnosis, although this association was not found for LINE-1 sequences [20].

Our methylation data was obtained using a method that directly measures the level of 5mC in the DNA
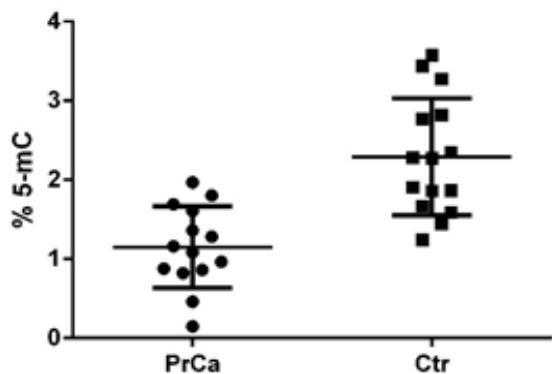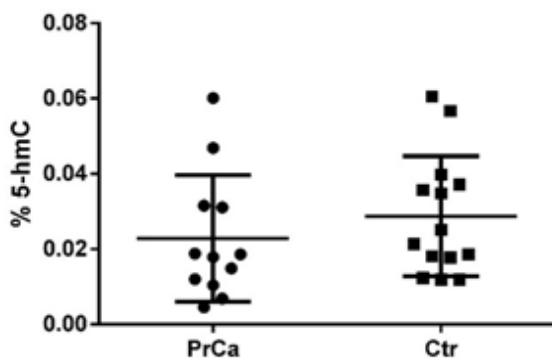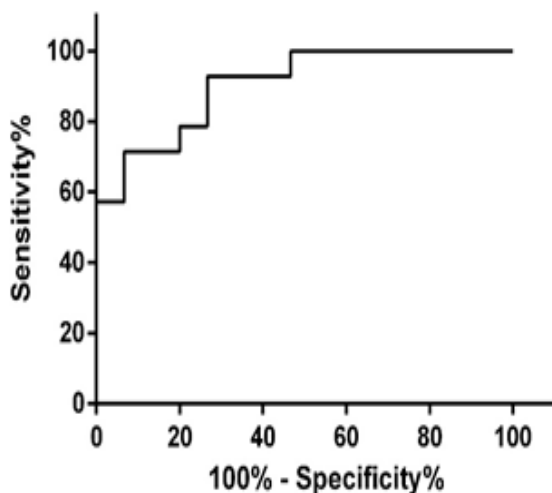
samples. Considering the pilot nature of our study, we found significant difference in global methylation levels of peripheral blood cells DNA in prostate cancer patients compared to controls. The lower level of methylation in patients compared to controls is in accordance with the global hypomethylation observed in prostate tissue studies compared to normal tissue. However, a major limitation of our study besides the small sample size is the age gap between cases and controls, a factor known to influence DNA methylation levels including in blood cells [21]. This issue would need to be addressed in future studies.

CONCLUSION

In this pilot study we found a significant difference in global methylation levels of peripheral bloodDNA from prostate cancer patients compared to controls. No difference was observed regarding the global hydroxymethylation levels. These findings need further replication in future larger studies in order to assess the biomarker potential of blood cells DNA methylation and hydroxymetylation in prostate cancer.

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